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A Simple In-Season Bioassay for Detecting Glyphosate Resistance in Grass and Broadleaf Weeds Prior to Herbicide Application in the Field

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The implementation of a successful glyphosate resistance management strategy requires a simple and cost-effective method for detecting resistance in key weeds. To date, however, glyphosate resistance is still routinely confirmed via laborious and time consuming whole-plant pot assays using seeds collected at the end of the growing season. Here, we describe a simple, early-season bioassay for detecting evolved glyphosate resistance in grass and broadleaf weeds. It involves transplanting suspected glyphosate resistant seedlings alongside known sensitive and resistant standards into agar containing informative rates of herbicide and recording percentage survival 14 d after plating. The method was validated using sensitive and resistant populations of *Lolium*, *Eleusine*, *Conyza*, and *Amaranthus* species encompassing the main glyphosate resistance mechanisms, namely, impaired translocation, EPSPS gene duplication, and mutations. The whole plant pot and agar-based seedling tests generated comparable resistance indices in dose-response assays and percentage survival at discriminating glyphosate rates. The method was applied successfully to detect resistance in a rigid ryegrass population collected from a French vineyard well before glyphosate was applied in the field for the current season. Additionally, the test was shown to be highly transferable to several other grass and broadleaf weeds that have evolved resistance to glyphosate. One major attribute of the method is that it is capable of detecting resistance regardless of the mechanism involved. In addition to being very simple, quick and, cost-effective, it allows determination of glyphosate resistance in weeds prior to field application. It thus offers the opportunity for an informed choice of herbicides for effective weed control.

Nomenclature: Glyphosate; horseweed, *Conyza canadensis* (L.) Cronq. ERICA; Italian ryegrass, *Lolium multiflorum* Lam. LOLMU; rigid ryegrass, *Lolium rigidum* (Gaudin); *Eleusine indica* (L.) Gaertn. ELEIN; common waterhemp, *Amaranthus rudis* J.D. Sauer; AMATA.

Key words: Weed resistance, agar-based bioassay, in-season resistance detection method.

Glyphosate has been hailed as a once in a century herbicide thanks to a set of very favorable attributes (Duke and Powles 2008). It is a broad spectrum herbicide which is efficacious on over 100 annual and 60 perennial weeds (Jaworski 1972). It is taken up rapidly by plants and moves efficiently to the growing points where it is most effective (Franz et al. 1997). It is safe for the environment and is characterized by low mammalian toxicity (Baylis 2000). When it was first commercialized in 1974, glyphosate was mainly employed for pre-plant burn-down and directed application in crops (Owen and Zelaya 2005). The use of glyphosate has increased substantially following the development of glyphosate resistant technology allowing for in-crop application. In the United States, a large proportion of all cotton, corn, and soybean acreage are planted to glyphosate resistant crops (Duke and, Powles 2009).

Glyphosate inhibits 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19) and depletes plants of essential aromatic acids, L-phenylalanine, L-tyrosine, and L-tryptophan (Steinrücken and Amrhein 1980). Collaterally, disruption of the shikimate pathway results in the accumulation of shikimic acid to toxic levels leading to plant death. As glyphosate is not significantly metabolized by plants, and because alteration of its binding site is often accompanied by a fitness penalty, glyphosate resistance evolution in weeds was deemed to be very unlikely (Sammons et al. 2007). For over 20 yr of use, glyphosate resistance was not documented. However, overreliance on glyphosate has led to the evolution of resistance in at least 28 weed species following a first report in a rigid ryegrass population from an orchard in Australia (Pratley et al. 1996). Currently, the most documented cases of glyphosate resistant weeds are

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from fields planted to glyphosate resistant crops (Heap 2014). In the USA alone, several million hectares of crop land are affected by glyphosate resistance. The most problematic weeds include *Amaranthus* spp., which are prone to accumulate resistance to multiple herbicide modes of action (Culpepper et al. 2006; Tranel et al. 2011).

Three major mechanisms account for glyphosate resistance in weeds, namely, exclusion of glyphosate from its main target, target gene duplication, and mutations (Perez-Jones and Mallory-Smith 2010; Powles and Yu 2010). In all cases, the level of resistance is relatively low, in the range of 3- to 15-fold, making confirmation of glyphosate resistance difficult in some cases (Shaner 2010). While resistance is a cause of concern and is constantly increasing, a significant proportion of weed species and populations around the world can still be effectively managed with glyphosate. To protect this precious technology, proactive use of multiple herbicide modes of action combined with nonchemical weed control measures are recommended. Equally important is the ability to confirm resistance as early as possible so that remedial actions can be taken to limit its spread.

Several glasshouse and laboratory-based methods have been described for detecting resistance to glyphosate in weeds (Burgos et al. 2013; Shaner 2010). To date, however, resistance is still confirmed in a vast majority of cases by collecting seeds at the end of the growing season and testing these alongside a standard sensitive population under controlled glasshouse conditions (Beckie et al. 2013; Gaines et al. 2012; Vink et al. 2012). This is a tedious and lengthy process that can take up to eight weeks and requires significant glasshouse space. Alternative testing methods include Petri-dish seed assay (de Carvalho et al. 2011; Perez-Jones et al. 2007; Zelaya and Owen 2005), HPLC and spectrophotometric analysis of shikimic acid from leaf discs (Koger et al. 2005; Mueller et al. 2003; Nol et al. 2012), lab-based DNA (Gaines et al. 2010; Kaundun et al. 2008; Ng et al. 2004), and physiological methods (Dinelli et al. 2006; Nandula et al. 2013; Perez-Jones et al. 2007) when resistance is associated with the target enzyme and impaired transport respectively. However, none of these latter techniques are used on a routine basis either because of germination issues, the requirement of sophisticated laboratory equipment or kits, or because they can only detect a subset of resistance mechanisms.

In this paper, we describe an alternative early-season assay for glyphosate resistance detection in grass and broadleaf weeds. The assay is adapted from the Syngenta RISQ (Resistance In-Season Quick) test that was initially developed for detection of grass weed resistance to acetolactate synthase and acetyl-CoA carboxylase inhibiting herbicides (Kaundun et al. 2010).

Materials and Methods

Seed Sources. The origins and characteristics of the species and populations used in this study are summarized in Table 1. Three sensitive and five resistant *Lolium* spp. populations and a sensitive and resistant each of common waterhemp, horseweed, and goosegrass were used to validate the method.

To determine whether the methodology was transferable to a wider range of grass and broadleaf weeds, one each of a sensitive population encompassing all the genera that have evolved resistance to glyphosate were also assayed. The species tested were common ragweed (*Ambrosia artemisiifolia* L.), ripgut brome (*Bromus diandrus* Roth), windmillgrass (*Chloris truncata* R. Br.), *Cynodon hirsutus* Stent, sourgrass [*Digitaria insularis* (L.) Mez ex Ekman], junglerice [*Echinochloa colona* (L.) Link], kochia [*Kochia scoparia* (L.) Schrad.], Judd's grass [*Leptochloa virgate* (L.) P. Beauv.], ragweed parthenium (*Parthenium hysterophorus* L.), annual bluegrass (*Poa annua* L.), johnsongrass, *Sorghum halepense* (L.) Pers., and liverseedgrass (*Urochloa panicoides* Beauv.).

Dose Responses in Whole Plant Pot Test. Seeds from the sensitive and resistant ryegrass populations were sown in trays containing peat and compost in a 1 : 1 ratio, covered with vermiculite and grown for 10 d in a glasshouse at 24/16 C day/night, 16-h day, 65% relative humidity, and a photon flux density of approximately 250 µmol quanta m^{-2} s^{-1}. One seedling was transplanted per 3 inch pot with the same soil media and grown to the two-leaf stage as described above.

Fifty individual plants were sprayed per glyphosate (Touchdown Total, Syngenta, NC) rate. Herbicide was applied using a cabinet track sprayer with a Teejet 11002VS nozzle delivering 200 L ha^{-1} spray volume. Percentage survival was assessed 21 d after treatment. The rates of herbicide applied are provided in Table 1.

Given the large number of ryegrass plants involved in this study, the populations were separated into three lots and tested in three separate experiments. In all three experiments a standard

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Table 1. Characteristics of the populations tested and herbicide rates used in the whole plant pot and agar-based seedling method.

Test	Population	Species	Origin	Characteristics	Whole plant pot test application rates (g ae ha^{-1})	Syngenta RISQ test concentrations (µM)
Lolium test 1	**(L1-S)**	*Lolium multiflorum*	UK, Herbiseed	Sensitive	0, 25, 50, 100, 200, 400, 800	0, 1.25, 2.5, 5, 10, 25, 50, 100
	L2-S	*Lolium multiflorum*	UK, Herbiseed	Sensitive	0, 25, 50, 100, 200, 400, 800	0, 1.25, 2.5, 5, 10, 25, 50, 100
	L3-R	*Lolium rigidum*	South Africa	Resistant - P106L target site	0, 25, 50, 100, 200, 400, 800	0, 1.25, 2.5, 5, 10, 25, 50, 100
Lolium test 2	**(L1-S)**	*Lolium multiflorum*	UK, Herbiseed	Sensitive	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
	L4-R	*Lolium rigidum*	South Africa	Mechanism not determined	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
	L5-R	*Lolium multiflorum*	Chile	Resistant - Impaired translocation	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
	L6-S	*Lolium rigidum*	Saudi Arabia	Sensitive	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
Lolium test 3	**(L1-S)**	*Lolium multiflorum*	UK, Herbiseed	Sensitive	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
	L7-R	*Lolium rigidum*	South Africa	Resistant - Low levels	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
	L8-R	*Lolium multiflorum*	Chile	Resistant - Impaired translocation	0, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 10, 25, 50, 100, 200, 500
Conyza	C1-S	*Conyza canadensis*	USA	Sensitive	0, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 0.1, 0.2, 0.45, 1, 2, 4.5, 10, 20, 44, 75
	C2-R	*Conyza canadensis*	USA	Resistant - Impaired translocation (unpublished data)	0, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 1, 2, 4.5, 10, 20, 44, 75, 94, 150, 200
Eleusine	E1-S	*Eleusine indica*	Philippines	Sensitive	0, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 5, 6.8, 9.7, 14, 20, 27, 38, 53, 72.2, 100
	E2-R	*Eleusine indica*	Philippines	Resistant - Pure SS106 target site	0, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512
Amaranthus	A1-S	*Amaranthus rudis*	Herbiseed, UK	Sensitive	0, 3.1, 6.3, 12.5, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 3.1, 6.3, 12.5, 25, 50
	A2-R	*Amaranthus rudis*	MN, USA	Resistant - *EPSPS* duplication (unpublished data)	0, 3.1, 6.3, 12.5, 25, 50, 100, 200, 400, 800, 1,600, 3,200, 6,400	0, 0.38, 0.75, 1.5, 3.1, 6.3, 12.5, 25, 50, 100, 200, 400

sensitive population (L1-S) was included, providing information on test-to-test variation.

The goosegrass populations were grown and tested with exactly the same method as the *Lolium* spp. populations. The common waterhemp and horseweed populations were sown in a similar way, except that they were grown at 24/18 C day/night, 16-h day. The common waterhemp and horseweed populations were sprayed either when the plants had reached a height of 8 cm or when the rosette was 10 cm in diameter.

Dose Responses in Agar-Based Syngenta RISQ Test. Two types of plastic agar plates were prepared to accommodate the different growing habits of the weed species tested. In all cases, plant agar (Duchefa) at 0.008 g ml^{-1} was melted in a microwave and allowed to cool to around 50 C before glyphosate (Touchdown Total, Syngenta, NC, USA) was added at the required concentrations. For all grass weed species and horseweed, agar was poured to a depth of 0.6 cm in 10 cm^2 Petri-dishes (Fisher Scientific Ltd) and allowed to set prior to transplanting. *Lolium* spp., goosegrass and horseweed seedlings were uprooted, cleaned from soil with water and placed horizontally on top of the agar. Using a pair of tweezers, the base of the stem was gently pushed into the agar with the remaining roots spread onto and making contact with the agar. Five seedlings were transplanted per Petri-dish, which was covered with a lid and placed in the same glasshouse conditions as for the whole plant pot assays.

For common waterhemp, M&S basal salts (Sigma Aldrich, UK) at 0.53 g L^{-1} were supplemented to the melted agar before glyphosate was added. The agar was poured to the brim of the square Petri-dishes and covered with a black plastic film to minimise water evaporation. A scalpel was used to make a thin slit into the plastic film and agar. Common waterhemp seedlings grown to a height of 8 cm were transplanted vertically into the agar by gently pushing the base of the plant through the slit. Ten plants were transplanted per Petri-dish and five replicate Petri-dishes (50 plants in all) were used per glyphosate rate.

Irrespective of the weed species and transplanting method, survival was assessed 14 d after transplanting, based on the development of healthy new shoots for common waterhemp and healthy shoots and roots for *Lolium* spp., goosegrass and horseweed. Typical agar-based RISQ test profiles at the discriminating rate of glyphosate are provided in Figure 1.

Application to a Field Sample. The newly-optimised agar-based RISQ test was applied on a known glyphosate resistant rigid ryegrass population from a French vineyard located in the 'Department de L'Herault'. In the previous season glyphosate efficacy averaged 60% in field trial plots in that vineyard. Plants at the one- to two-leaf stage were collected in October 2012 and stored in moist tissue paper at 10 C overnight. The next day, they were transplanted into agar containing glyphosate at rates of 0, 25, 50, and 100 μM. Fifty individual seedlings were transplanted per herbicide rate. In the absence of a glasshouse, the Petri-dishes were placed at a window sill in natural daylight and room temperature. The standard sensitive population L1-S and L5-R were grown and tested alongside for comparison. Survival was assessed based on the development of new shoots and roots, 14 d after transplanting.

Transferability of the Method to a Wide Range of Weed Species. Twelve weed species comprising all the other genera that have evolved resistance to glyphosate were assayed. The sensitivity of the populations was confirmed based on the commonly used rate of glyphosate at 840 g ae ha^{-1} in whole plant pot tests. Ten individually potted plants or two replicates of five plants per pot were tested for broadleaf and grass weeds respectively.

To determine whether the species were amenable for testing in the agar-based methodology, 10 plants were tested per species and glyphosate rate. For all weeds, two replicates of five plants were tested per Petri-dish. The rates chosen (25, 50 and 100 μM) were around the discriminating rates identified for the two grass (*Lolium* spp. and goosegrass) and two broadleaf weeds (horseweed and common waterhemp) tested in the dose-response assays.

Statistical Analysis. Data for each dose response test on both the whole plant and agar-based seedling platforms were analyzed separately. Since percentage survival is based on the number of plants surviving out of a known number tested, the relationship between percentage survival and log(rate) was analyzed by iteratively re-weighted least squares regression ('logit/probit' analysis) with a common slope fitted to the regression lines for each biotype (Finney 1971). This was generally found to be adequate (Figure 2) for the purposes of estimating LD_{50}s (the rate which causes a 50% reduction in survival) and resistance indices (RIs) between each biotype and the designated sensitive biotype in the

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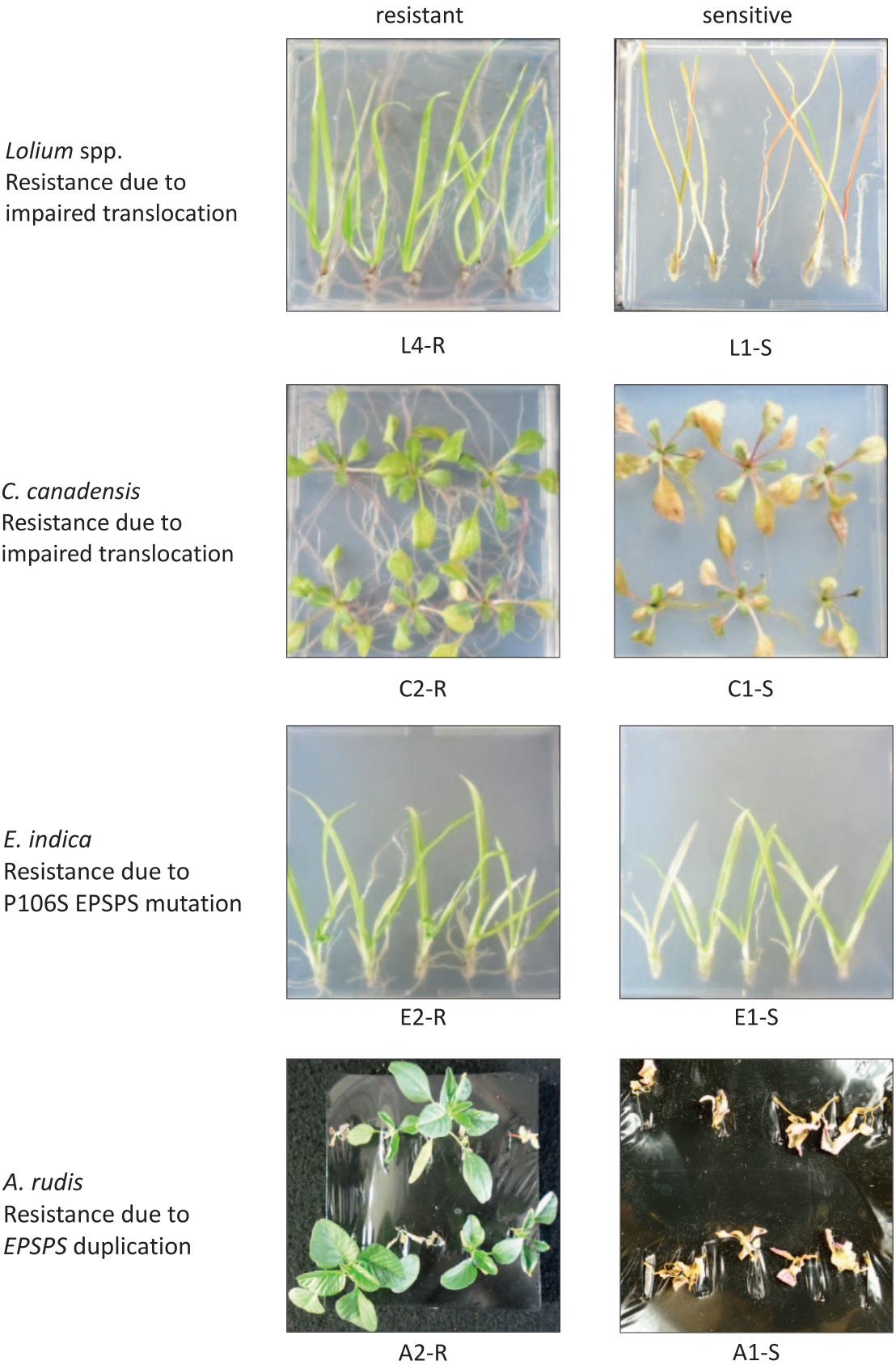

Figure 1. Typical resistant and sensitive weed populations assayed at the discriminative rate of 50 μM glyphosate in the agar based RISQ test.

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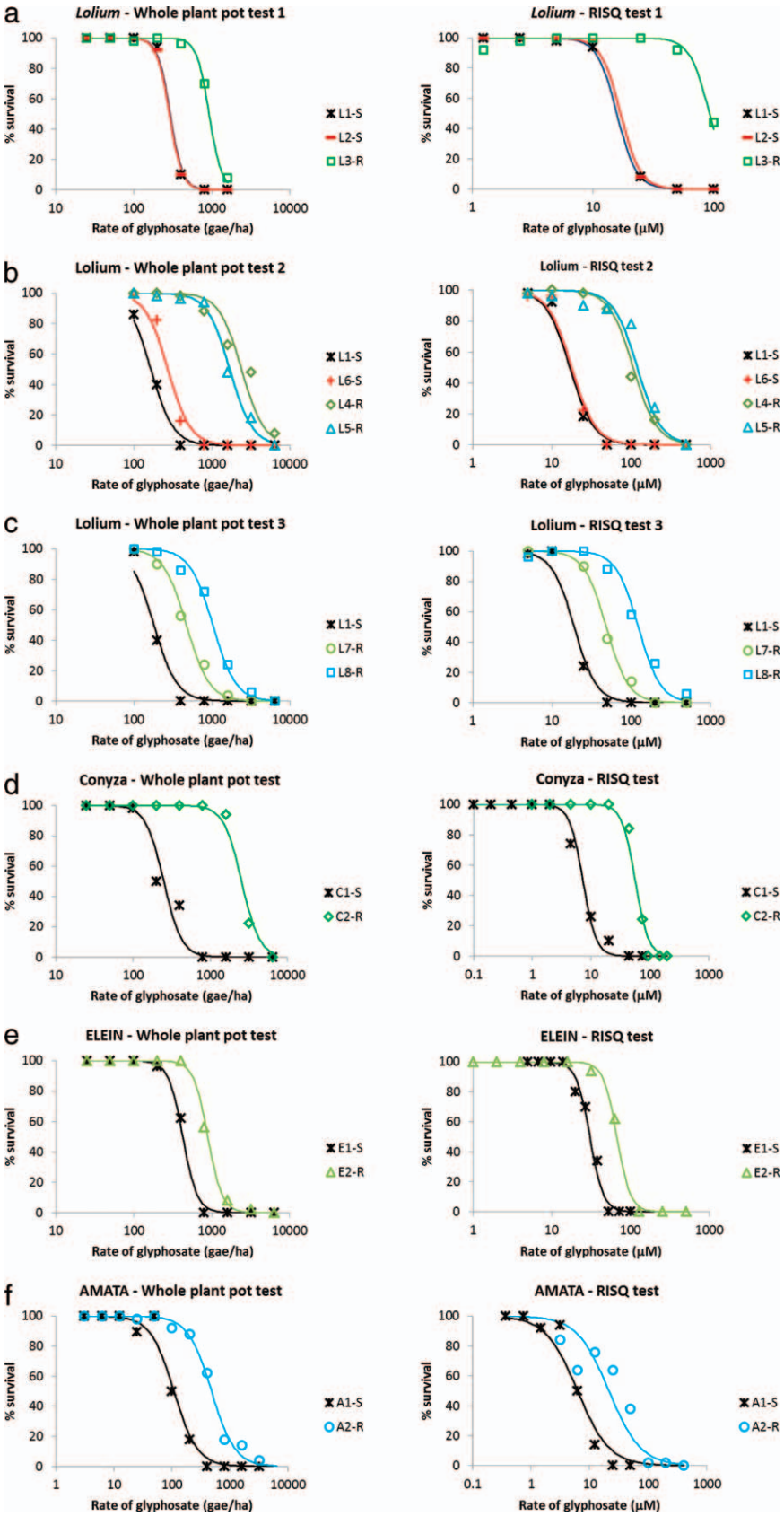

Figure 2. Glyphosate dose responses in whole plant pot- and agar-based RISQ tests: (a) *Lolium* spp. test 1, (b) Lolium spp. test 2, (c) Lolium spp. test 3, (d) horseweed, (e) rigid ryegrass, and (f) common waterhemp.

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Table 2. Estimated LD_{50} values and resistance indices by the two methods.

Test	Population	Pot test		Agar test	
		Estimated LD_{50} (95% confidence limits)	Estimated resistance index (95% confidence limits)	Estimated LD_{50} (95% confidence limits)	Estimated resistance index (95% confidence limits)
Lolium, test 1	L1-S	292 (262–324)		15.8 (13.8–18.1)	
	L2-S	287 (258–319)	0.98 (0.85–1.14)	17.0 (14.8–19.4)	1.08 (0.89–1.30)
	L3-R	922 (836–1,020)	3.16 (2.74–3.66)	92.5 (83.7–102.3)	5.85 (4.94–6.93)
Lolium, test 2	L1-S	165 (143–190)		16.9 (12.2–23.2)	
	L4-R	2408 (2,097–2,767)	14.58 (11.97–17.82)	106.2 (78.6–144.0)	6.30 (4.07–9.85)
	L5-R	1648 (1437–1,892)	9.98 (8.20–12.19)	119.5 (89.2–161.1)	7.09 (4.61–11.04)
	L6-S	273 (238–314)	1.66 (1.36–2.02)	17.7 (12.8–24.3)	1.05 (0.67–1.65)
Lolium, test 3	L1-S	181 (157–208)		19.1 (16.4–22.2)	
	L7-R	472 (412–541)	2.61 (2.15–3.18)	48.4 (42.3–55.3)	2.53 (2.08–3.10)
	L8-R	1027 (896–1,177)	5.68 (4.68–6.91)	122.7 (107.3–140.5)	6.42 (5.26–7.88)
Conyza	C1-S	249 (141–448)		7.2 (6.4–8.2)	
	C2-R	2536 (1420–4,539)	10.2 (4.5–23.0)	55.8 (50.2–61.5)	7.69 (6.52–9.03)
Eleusine	E1-S	421 (216–873)		30.5 (25.1–37.2)	
	E2-R	889 (462–1,904)	2.11 (0.80–5.83)	66.5 (50.7–87.8)	2.18 (1.56–3.06)
Amaranthus	A1-S	108 (72–163)	4.43 (2.53–7.70)	6.3 (3.8–10.5)	3.43 (1.64–7.05)
	A2-R	479 (328–700)		21.5 (12.7–35.8)	

same test, estimated as the ratio of their respective LD_{50} estimates.

Data comparing survival of the field sample to the known sensitive L1-S population were analyzed using Fisher's exact test separately at each of the glyphosate rates. A p-value of 0.05 or less is customarily regarded as indicating a statistically significant result.

Results and Discussion

Whole Plant Pot Test. Given the large number of ryegrass populations and herbicide doses involved, the tests were conducted in three batches (Table 1). All plants in population L1-S were controlled at 800 g ha^{-1} glyphosate in the first test and 400 g ha^{-1} in the second and third tests, reflecting test-to-test variations that can occur with this and other herbicides (Figure 2a). It is to be noted that only 10% of plants survived at 400 g ha^{-1} glyphosate for the first test. Calculated LD_{50} values for L1-S were 292, 165 and 181 g ha^{-1} glyphosate in each of the three tests (Table 2). In two other sensitive populations, L2-S and L6-S, all plants were killed at 800 g ha^{-1} with resistance indices of 0.98 and 1.66, respectively when compared to L1-S, thus indicating similar sensitivities to glyphosate for all three populations. At the discriminating rate of 800 g ha^{-1} glyphosate, survival of the five resistant populations ranged from 24% to 94%. The associated resistance indices varied from 2.6 for L7-R to 14.6 for L4-R through 5.7 for L8-R (Figure 2a, Table 2). These indices were in line with previous studies for the populations for which the mechanism of resistance were known, namely, a target site mutation (P106L) and other minor mechanisms for population L3-R (Collavo and Sattin 2012; Kaundun et al. 2011; Wakelin and Preston 2006), and impaired glyphosate translocation for populations L8-R and L5-R (Perez-Jones et al. 2007; Preston and Wakelin 2008). On the basis of the resistance indices estimated for L4-R and L7-R from South Africa, impaired translocation and a gene mutation are inferred, though proper physiological and molecular tests are needed to confirm the resistance mechanisms in these populations.

Plants in the sensitive goosegrass sub-population E1-S were all controlled at 800 g ha^{-1} glyphosate. At this rate, survival of the corresponding pure homozygous mutant subpopulation E1-R was 56%. The estimated resistance index was 2.1 between the wild type and EPSPS mutant sub-populations in accord with the low and partial resistance conferred by the P106S mutation in goosegrass (Baerson et al. 2002; Kaundun et al. 2008). In contrast, all the plants from the glyphosate resistant horseweed population C2-R, characterized as resistant through impaired herbicide translocation, survived at the rate (800 g ha^{-1} glyphosate) that controlled the sensitive population. The estimated resistance index (10.2) was relatively high and in the range reported for horseweed populations with the impaired translocation mechanism (Koger et al. 2004; VanGessel 2001). An intermediate level of glyphosate resistance (RI of 4.4) was found for the common waterhemp population A2-R, characterized by gene amplification

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(Gaines et al. 2010; Mohseni-Moghadam et al. 2013). It is noteworthy that 62% of A2-R plants survived the rate of glyphosate that killed the sensitive population A1-S.

Agar-Based Syngenta RISQ Test. The standard, sensitive ryegrass population, L1-S was consistently killed at 50 μM glyphosate across the three tests (Figure 2b). LD_{50} values for L1-S were very comparable among the three tests, estimated at 15.8, 16.9 and 19.1 μM glyphosate (Table 2). The two additional sensitive populations L2-S and L6-S also required 50 μM glyphosate for effective control (Figure 2b). The estimated resistance indices for the two sensitive populations L2-S and L6-S were 1.08 and 1.05, respectively, with the value 1 within confidence intervals, indicating that these were as sensitive to glyphosate as the standard sensitive population, L1-S. At the discriminating dose of 50 μM, the survival rate ranged from 42–92% for the five resistant populations. The associated resistance indices varied from 2.5 for population L7-R to 7.1 for population L5-R (Table 2). Higher levels of resistance were observed for populations L5-R and L8-R, with confirmed impaired glyphosate translocation mechanism, than for population L3-R, with an EPSPS target site mutation and some other minor underlying mechanism(s) (Kaundun et al. 2011). The wild type PP106, goosegrass subpopulation was controlled at 53 μM glyphosate whilst the mutant SS106, subpopulation required 128 μM glyphosate for complete kill. The difference in response of the two goosegrass populations was small, with an estimated resistance index of 2.2 (Table 2), in agreement with the level of resistance conferred by an EPSPS mutation at codon position 106 (Kaundun et al. 2008). When compared with the respective sensitive populations, intermediate and high levels of resistance were found for the common waterhemp population A2-R (3.43) and horseweed populations C2-R (7.69) characterised by gene amplification and impaired translocation, respectively (Table 2). It is noteworthy that the sensitive and resistant horseweed populations could be clearly differentiated despite the small plants used. This contrasts with previous reports demonstrating that plant size at application can have a profound influence on the level of glyphosate resistance in this species (Shrestha et al. 2007). Indeed, under glasshouse conditions, resistant plants at the rosette stage could still be killed by the recommended rate of glyphosate (Dinelli et al. 2006).

Table 3. Survival at selected, discriminating rates in the whole plant pot and agar-based seedling tests.

	Survival at discriminating rate (%)	
Lolium population	Pot test (800 g ae ha^{-1})	Agar-based RISQ test (50 μM)
L1-S	0	0
L2-S	0	0
L3-R	70	92
L1-S	0	0
L4-R	88	88
L5-R	94	88
L6-S	0	0
L1-S	0	0
L7-R	24	42
L8-R	72	88

Comparison of Whole Plant Pot and Agar-Based Test Results. The resistance indices for the ryegrass populations estimated with the established whole plant, pot and newly developed Syngenta RISQ test were very comparable and with the same order of magnitude for the two methods (Table 2). This was particularly apparent for the two sensitive populations, L2-S and L6-S, as well as the resistant populations, L7-R and L8-R. Overall, the RISQ test could clearly differentiate between the three sensitive and five resistant populations, including L3-R and L7-R that are characterized by low levels of resistance (Figures 2a and 2b). It is notable that the resistance indices were not consistently higher for the pot test than the RISQ test, indicating that the newly developed method was not missing minor mechanisms that could be expressed in the whole plant pot, but not the RISQ test. At the rate that killed all sensitive populations in the whole plant pot and agar-based assay, survival was very comparable for all populations in the two methods (Table 3).

The resistance indices estimated with the two methods were also very comparable for the horseweed, goosegrass, and common waterhemp populations characterized by high, medium and low levels of resistance (Table 2). Survival around the discriminating dose of glyphosate was very comparable with the two methods, further attesting to the robustness of the newly-optimized RISQ test for detecting glyphosate resistance in these three species. The RISQ test data from this study, on a limited number of populations, suggest that discrimination between the sensitive and resistant phenotypes can be achieved at a single rate of 50 μM. However, given the possible greater variation between field populations, the relatively low levels of resistance generally associated with glyphosate (Sammons et al. 2007), and the inherent

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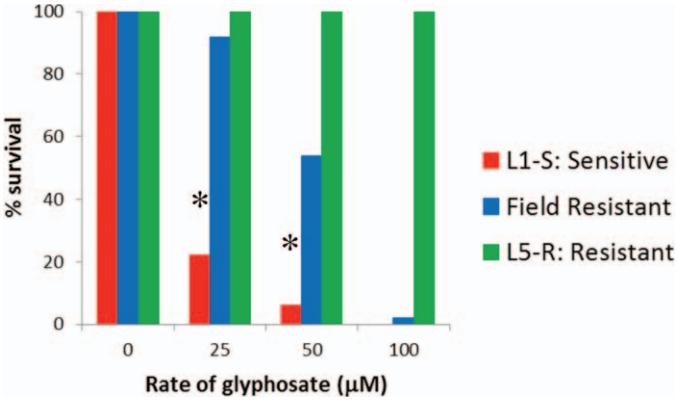

Figure 3. Percentage survival of field collected plants relative to sensitive (L1-S) and resistant (L5-R) standards. (*): denotes a statistically significant result between the standard sensitive and field samples ($P < 0.0001$) as analyzed by the Fisher's Exact test.

variability of glyphosate responses for some species (Westhoven et al. 2008; Zelaya and Owen 2005), it may be unrealistic to expect a single specific rate of glyphosate to confidently distinguish between resistant and susceptible phenotypes in any particular situation. The best approach, therefore, would be to test several rates in order to build a rate-response relationship for each population, provided that a sufficiently large sample of seedlings was tested at each rate. Where resources are limited, three rates of 0.5X, X, and 2X may be adequate, where X represents the most likely *a-priori*, discriminating rate between sensitive and resistant populations in a given situation.

Application to Field Samples. A higher proportion of field-collected plants survived at all three rates of glyphosate tested as compared to the standard sensitive population L1-S. Analysis of the data with the Fisher's Exact test identified a significant difference at 25 µM ($p < 0.0001$) and 50 µM ($p < 0.0001$) between the field and standard sensitive samples thus confirming the resistance status of the ryegrass population from the French vineyard (Figure 3). In contrast to the results of numerous tests carried out in the glasshouse, three out of fifty sensitive L1-S plants survived at the previously established discriminating rate of 50 µM glyphosate. Similarly, a larger proportion of the standard resistant population L5-R survived as compared to glasshouse conditions. The overall higher levels of survival across the different rates and standard populations may be due to the sub-optimal light conditions at the windowsill test. This is not surprising, as herbicides are most effective on rapidly-growing, healthy plants (Burgos et al. 2013). Nevertheless, the resistance status of the ryegrass population could be determined early enough in October to allow planning for effective weed management in the vineyard in April the following year.

Transferability to Other Weed Species. As expected, all the plants belonging to 12 genera were controlled at the commonly used rate of 840 g ha^{-1} in the whole plant pot test as they were from sensitive populations only. The species, encompassing four broadleaf and eight grass weeds, grew well in the untreated plates, demonstrating their amenability for testing in the agar-based RISQ method. All but one population required 50 µM glyphosate for complete kill. The exception was ripgut brome, which was controlled at the lower rate of 25 µM. As with the ryegrass populations used in this pilot study, a larger number of sensitive and resistant populations characterized by different types and frequencies of resistance should be assayed to determine more robust, discriminating and informative rates for each species.

Comparison with Existing Method. The agar-based seedling assay is similar to the whole plant pot test in that it can detect resistance, regardless of the mechanism involved. However, the major difference is the treatment of seedlings prior to herbicide treatment in the field, rather than waiting for seed collection at the end of the growing season. This represents a major advantage over resistance tests that are carried out post-herbicide application. Indeed, product failure in a season can result in significant yield losses and associated exacerbation of the glyphosate resistance problem in subsequent years. Additionally, as the Syngenta RISQ test uses seedlings collected from the field, it is not affected by seed dormancy and germination issues, as is the case for tests starting with seeds. Furthermore, the seedling-based agar assay requires limited glasshouse space compared to whole plant pot tests and does not require watering or any other plant maintenance, once the seedlings are transplanted into agar. A sprayer is not required since glyphosate is formulated into the agar.

DNA analyses of known target site EPSPS resistance mutations (Kaundun et al. 2008; Ng et al. 2004) or duplication (Gaines et al. 2010) are also applicable prior to herbicide application in the field. However, these DNA-based methods will neither detect mutations yet to be uncovered nor identify resistance due to impaired translocation, which is

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the predominant mechanism of resistance in *Conyza* and *Lolium* species (Preston and Wakelin 2008). Likewise, ^{14}C-glyphosate, translocation assays require expensive laboratory kits, as well as skilled and licensed personnel to carry out radiolabelled work. Whilst attractive and fast, demonstration of the applicability of the excised-leaf, necrotic assay, initially validated for horseweed, to a range of populations and species is needed (Koger et al. 2005). The same is true for the enzyme-based colorimetric assay under development for detecting glyphosate resistance in the field (Sammons et al. 2013).

The primary concern regarding the diagnostics value of the agar-based RISQ test assay developed here is that test results may not always be generated in time for those species characterized by rapid growth under certain latitudes and agronomic conditions. This may be addressed by sampling and assaying early germinators from the field. It may also be an issue for species, such as horseweed, which have been reported to manifest resistance primarily at later growth stages. However, as demonstrated here, the sensitive and resistant horseweed populations could be clearly identified, even when small plants were used in the agar-based seedling test.

Contribution to Glyphosate Resistance Management. It is now widely recognized that inevitably, weeds subjected to herbicide pressure or any other selection agent, will find ways to evolve resistance to allow survival of the species, thanks primarily to their high genetic plasticity. This process may be slower for some herbicides, such as glyphosate, due to intrinsic characteristics linked to its mode of resistance and efficient kill at the recommended field rates. Nonetheless, it is expected that practitioners will take a long term, sustainable approach by rotating crops and herbicide mechanisms of action, combined with implementation of nonchemical weed control methods to delay the onset of resistance. However, the current reality is different and in most cases, integrated weed management strategies are adopted only when resistance has been confirmed in the field in question. Therefore, simple, proactive, and cost-effective tests, such as the one described here, are critical for detecting resistance as early as possible, so that corrective measures can be taken in time to limit the spread of resistance. This is particularly important for highly prolific species such as *Amaranthus* spp. that can render a field nonfarmable in only 3 yr if not controlled (Norsworthy 2013). Examples of where the test can be effectively applied include determination of the resistance status of the ever-expanding Palmer amaranth populations in the northern states of the United States, as well as an investigation of the recent appearances of the same species in soybean production systems in Argentina. As the method has also shown to be widely amenable to a range of diverse grass and broadleaf weeds, it can also be applied in species that have become more tolerant and will lead to glyphosate failure in the near future. In conclusion, we have developed a very simple and widely applicable method that can determine the glyphosate resistance status of weed populations prior to herbicide application in the field and, therefore, can be valuable in sustaining the use of glyphosate.

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